

Ultra-thin layer MALDI mass spectrometry of membrane proteins in nanodiscs

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Abstract Nanodiscs have become a leading technology to solubilize membrane proteins for biophysical, enzymatic, and structural investigations. Nanodiscs are nanoscale, discoidal lipid bilayers surrounded by an amphipathic membrane scaffold protein (MSP) belt. A variety of analytical tools has been applied to membrane proteins in nanodiscs, including several recent mass spectrometry studies. Mass spectrometry of full-length proteins is an important technique for analyzing protein modifications, for structural studies, and for identification of proteins present in binding assays. However, traditional matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry methods for analyzing full-length membrane proteins solubilized in nanodiscs are limited by strong signal from the MSP belt and weak signal from the membrane protein inside the nanodisc. Herein, we show that an optimized ultra-thin layer MALDI sample preparation technique dramatically enhances the membrane protein signal and nearly completely eliminates the MSP signal. First-shot MALDI and MALDI imaging are used to characterize the spots formed by the ultra-thin layer method. Furthermore, the membrane protein enhancement and MSP suppression are shown to be independent of the type of

membrane protein and are applicable to mixtures of membrane proteins in nanodiscs.

Keywords MALDI-TOF mass spectrometry · Membrane proteins · Nanodisc · Rhodopsin · Cytochrome p450 reductase · Cytochrome P450 3A4

Introduction

Membrane proteins are challenging to interrogate directly with many biophysical and biochemical methods due to their insolubility in aqueous systems. Over the last decade, nanodiscs have emerged as an effective tool for solubilizing membrane proteins for functional and structural studies [1–3]. Nanodiscs are homogeneous nanoscale lipid bilayers surrounded by amphipathic membrane scaffold protein (MSP) belts (Fig. 1). As detergent is slowly removed from the mixture, membrane proteins are incorporated into the lipid bilayer as the lipids, MSP, and membrane protein self-assemble into nanodiscs. Membrane proteins in nanodiscs are in a native-like lipid bilayer environment, which is more physiologically relevant than detergent-solubilized systems [4]. The analytical toolbox for membrane proteins in nanodiscs includes a wide variety of techniques such as solid-state NMR [5], electron microscopy [6–8], optical spectroscopy, and refractive-indexed-based plasmonic sensing [9–11].

Previously, membrane proteins in nanodiscs have been studied with several types of mass spectrometry. Hydrogen exchange mass spectrometry was used to investigate native conformations of membrane proteins in nanodiscs [12]. First, hydrogen–deuterium exchange was performed on γ -glutamyl carboxylase (GGCX) in nanodiscs. Then, the nanodiscs were disassembled and GGCX and MSP were protease-digested. Peptide fragments were examined with ultra-performance liquid chromatography-mass spectrometry to determine deuterium uptake rates. Although MSP peaks dominated the

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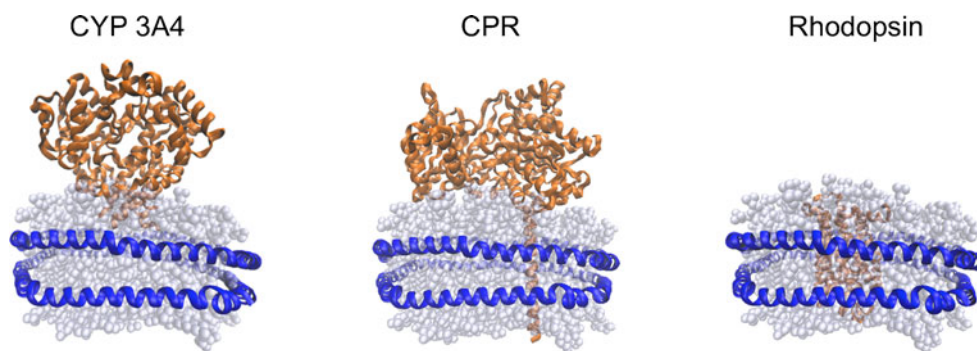


Fig. 1 Model of CYP 3A4, CPR, and rhodopsin in nanodiscs. The two MSP molecules are shown in *blue*, proteins are shown in *orange*, and the POPC lipid bilayer is shown in a *translucent light blue*. CYP 3A4 is

monotopically embedded in the membrane, CPR is associated with the membrane through a single transmembrane helix, and rhodopsin is polytopically buried in the membrane

chromatograms and mass spectra, control experiments with lipid-only nanodiscs allowed subtraction of the MSP contribution. Hydrogen exchange mass spectrometry of membrane proteins in nanodiscs allows for unique structural studies of membrane proteins accounting for interactions with the lipid bilayer.

In another work, mass spectrometry in conjunction with co-immunoprecipitation has been used to identify enterotoxins binding to glycolipid ganglioside GM1 in nanodiscs [13]. Agarose beads immobilized with an antibody to capture nanodiscs were incubated with GM1 nanodiscs and *Escherichia coli* cell cultures expressing enterotoxins. After washing and eluting from the agarose beads, captured enterotoxins and MSP were separated by gel electrophoresis, digested with trypsin, and identified by MALDI-TOF mass spectrometry. While this study examined glycolipids in nanodiscs rather than membrane proteins, it illustrates the potential of nanodisc co-immunoprecipitation mass spectrometry assays for identifying proteins involved in binding to targets in nanodiscs.

Mass spectrometry methods relying on protease digests are valuable in a variety of studies as exemplified above. However, mass spectrometry of full-length proteins is also an important analytical technique. There is one example of a study utilizing self-assembled monolayers for matrix-assisted laser desorption ionization (SAMDI) that observed full-length proteins in nanodiscs. In this study, nanodiscs containing rhodopsin, a photo-activated G protein-coupled receptor, were captured on a self-assembled monolayer (SAM) surface [14]. After flowing transducin solution over the surface, matrix was applied to the dried surface, and MALDI-TOF mass spectrometry was performed directly on the SAM surface. Transducin binding was detected by mass spectrometry when rhodopsin was activated by light but not observed when the assay was performed in the dark. Although this assay focused on transducin, a soluble protein, rhodopsin was visible in the spectrum, which helped to confirm its successful capture on the monolayer. This SAMDI assay demonstrated the potential of mass spectrometry with nanodiscs as a sensitive functional assay.

There is great potential for further study of full-length proteins in nanodiscs using mass spectrometry. In addition to functional assays such as the SAMDI assay described above, interactions involving proteins complexes and heterogeneous populations of nanodiscs could be probed in novel binding experiments. In general, precise values of protein molecular weights can be used to identify inhomogeneity in protein samples, the presence of post-translational modifications, or the efficacy of label attachment [15–18]. Mass spectrometry following limited proteolysis can give key structural information and has become important in identifying domains for crystallization [19, 20]. Finally, post-ionization fragmentation of proteins can be used for protein investigation with top-down proteomics [21, 22]. Nanodiscs offer the unique ability to perform these experiments on membrane proteins in a soluble, native-like lipid bilayer.

However, as observed in the SAMDI assay and in data presented here, conventional MALDI sample preparation techniques give weak membrane protein peaks and strong MSP peaks, which complicate the spectrum and overwhelm the signal observed from the membrane protein of interest. This is not a surprising problem. Membrane proteins have been difficult to study using mass spectrometry due to their poor solubility in many common solvents and the deleterious effects of many detergents on the spectra. Since lipids and MSP are present in nanodiscs at least at a 130:2:1 lipid/MSP/membrane protein ratio, membrane proteins in nanodiscs are in relatively low abundance and are in the presence of high lipid concentrations. There are several sample preparation methods that have been developed to improve detection of membrane proteins in detergent and that could potentially be applied to nanodisc samples [15, 23]. However, sample preparation that involves chromatography is generally more time and material intensive.

In this study, we present an ultra-thin layer MALDI method that has been optimized for membrane proteins in nanodiscs. This method has been previously applied to MALDI of membrane proteins in detergent [17, 24, 25]. It relies on preparation of the sample plate surface with a thin layer of matrix, which acts as seed crystals to nucleate formation of

homogenous polycrystalline matrix layer on the surface. The speed and simplicity of this method have led to its wide adoption, especially as a partner to crystallographic studies of membrane proteins [20].

We apply the ultra-thin layer method to membrane proteins in nanodiscs and show that we can nearly entirely eliminate the MSP signal while providing a dramatic enhancement of the membrane protein signal. The method was tested on several structurally diverse proteins including (a) cytochrome P450 3A4 (CYP 3A4), which is monotonically embedded in the membrane, (b) cytochrome p450 reductase (CPR), which is membrane associated through a single hydrophobic transmembrane helix, and (c) rhodopsin (Rho), which is a polytopic integral membrane protein (Fig. 1). Furthermore, we use this method to identify multiple proteins in a heterogeneous mixture of membrane proteins in nanodiscs. This enhancement of membrane protein peaks and elimination of the scaffold protein allows fast and easy analysis of modifications to proteins in nanodiscs and opens the door for experiments involving structural studies, heterogeneous libraries of membrane proteins in nanodiscs, and novel mass spectrometry assays.

Experimental methods

Materials

Sinapinic acid (SA) was obtained from Sigma-Aldrich-Fluka and used without further purification, and α -cyano-4-hydroxycinnamic acid (4HCCA) was obtained from Sigma-Aldrich (St. Louis, MO) and recrystallized in ethanol. High-performance liquid chromatography-grade methanol, isopropanol, and acetonitrile as well as 88% formic acid and liquid chromatography–mass spectrometry-grade trifluoroacetic acid (TFA) were obtained from Fisher Scientific (Pittsburgh, PA). Water was purified with Millipore (Marlborough, MA) cartridges to 18.2 M Ω ·cm.

Palmitoyl-oleoyl-phosphatidylcholine (POPC) was obtained from Avanti Polar Lipids (Alabaster, AL), and octyl glucoside was obtained from Anatrace (Maumee, OH). Sodium cholate, Triton X-100, and Amberlite (XAD-2) were purchased from Sigma Chemical (St. Louis, MO). Emulgen 913 was from Karlan Research Products (Santa Rosa, CA).

Nanodisc preparation with membrane proteins CYP3A4, CPR, and rhodopsin

Membrane scaffold protein expression, purification, and nomenclature

CYP3A4, CPR, and rhodopsin were assembled into nanodiscs using MSP1D1 as the membrane scaffold protein.

MSP1D1 has been described previously in detail [3]. The original version of membrane scaffold protein, MSP1, is an engineered protein based on human apolipoprotein A-I and optimized for expression and purification from *E. coli* [1]. For MSP1D1, amino acids 1–11 in the first helix of MSP1, which do not participate in the scaffold protein–lipid interface, have been deleted. It has an engineered tobacco etch virus protease site to allow cleavage of the polyhistidine affinity tag used for purification. MSP1D1 was cloned into the pET vector, expressed in *E. coli* BL21-Gold (DE3) cells (Stratagene) and purified as described in [1]. Cleavage of the polyhistidine tag has been described previously [3]. MSP1D1 with the polyhistidine tag removed is referred to as MSP1D1(–) and is used with CYP 3A4 and CPR nanodiscs.

CYP3A4, CPR, and rhodopsin protein expression and purification

CYP3A4 with a pentahistidine affinity tag was expressed from the NF-14 construct in the pCWOri+vector and purified as previously described [26, 27]. Rat CPR was expressed in the pOR262 vector and purified as described in Das and Sligar [28]. Bovine rhodopsin was purified by isolating rod outer segments from frozen bovine retina (SPCI Retina Inc.). Rhodopsin was purified from these rod outer segments on ConA-sepharose (GE Healthcare) as described in Vishnivetskiy [29].

Self-assembly of nanodiscs containing membrane proteins
cytochrome P450 3A4, CPR, and rhodopsin

The membrane protein nanodisc assembly is a three-component system consisting of MSP, the membrane protein target, and lipids. All the components are mixed and detergent-solubilized. As the detergent is removed with Amberlite XAD-2 beads (porous cross-linked polystyrene polymer beads that adsorb detergents), the system self-assembles to form nanodiscs. This general protocol for forming the membrane protein nanodisc is common to all membrane proteins, but there can be specific differences in the type of membrane scaffold protein and detergent used for different membrane proteins. The assembly of CYP 3A4 and CPR nanodiscs is described in detail in Baas et al., Denisov et al., and Grinkova et al. [26, 27, 30]. The assembly of rhodopsin nanodiscs is detailed in [31]. A brief description of the nanodisc assembly is given below.

The components used to self-assemble CYP3A4, CPR, and rhodopsin into nanodiscs were MSP, cholate-solubilized POPC, and purified membrane proteins initially solubilized in detergent. For CYP 3A4 and CPR, MSP1D1(–) was used. CYP 3A4 was initially solubilized in 0.1% (v/v) Emulgen 913, and CPR was initially solubilized in 0.1% (w/v) Triton

X-100. For rhodopsin, MSP1D1 was used, and rhodopsin was initially solubilized in 90 mM octyl glucoside. Membrane proteins were typically concentrated to around 100 μM before being mixed with the other components. MSP was prepared as described above and concentrated to around 200 μM . POPC in powder form was dissolved in chloroform, dried under nitrogen, and put in a vacuum desiccator overnight to remove all residual chloroform. The dried phospholipids were then dissolved in a buffer containing 100 mM cholate to a lipid concentration of 50 mM and sonicated to ensure solubilization of the phospholipids.

The cholate-solubilized POPC, detergent-solubilized membrane protein, and MSP were combined in a molar ratio of (1:65:130:0.1) (MSP/POPC/cholate/membrane protein). Typically, the final concentrations of the different components in the mixture were around 15.3 μM membrane protein, 153 μM MSP, 10 mM POPC, 20 mM cholate, and residual amounts of Emulgen 913, Triton X-100, or octyl glucoside. The excess of MSP in the assembly mixture ensures an excess of empty nanodiscs to statistically favor incorporation of only one membrane protein molecule per nanodisc. Thus, a single nanodisc would consist of two MSP1D1 molecules, 130 POPC molecules, and one membrane protein molecule. This mixture was incubated on ice for 1 h.

To initiate the self-assembly process, detergent was slowly removed from the mixture by 4 h of incubation on an orbital shaker at 4 °C with 0.5–0.8 g of Amberlite XAD-2 per milliliter of solution. Following removal of Amberlite beads from the solution by centrifugation, nanodiscs were purified from any lipid or MSP aggregates by size exclusion chromatography on a Superdex HR 200 10/30 size exclusion column (Amersham–Pharmacia Biotech, Piscataway, NJ). Fractions containing nanodiscs were pooled.

With rhodopsin and CPR, no further purification was performed to separate membrane protein-containing nanodiscs from the excess lipid-only nanodiscs. For CYP 3A4, the nanodiscs containing CYP 3A4 were isolated on a nickel nitrilotriacetic acid metal chelating column using the pentahistidine affinity tag on CYP 3A4 (see [26]). Finally, all nanodisc samples were concentrated to around 10 μM membrane protein concentration using a 10,000 Da molecular weight cut off centrifugal protein concentrator from Millipore.

MALDI sample preparation

For the dried-drop method of sample preparation, a matrix-saturated solution was prepared by adding solid matrix to 1:1 water/acetonitrile with 0.1% TFA and mixing thoroughly. Enough solid matrix was added so that some of the

matrix remained undissolved, and the solid matrix settled to the bottom of the tube. Nanodisc samples were diluted 1:10 in the matrix-saturated solution, and 1 μL was spotted directly onto a stainless steel 100-spot Voyager sample plate. The spot was allowed to dry in air.

The ultra-thin layer method relies on preparation of the sample plate with a thin layer of dried matrix [17, 24, 25]. The stainless steel 100-spot Voyager sample plate or ground steel 384-spot Bruker sample plate was washed thoroughly with multiple alternating methanol and water rinses. To prepare the ultra-thin layer solution, 150 μL of 1:500:500 *v/v/v* TFA/water/acetonitrile (TWA) was added to 10 mg of solid matrix. After thoroughly mixing at room temperature by repeated aspiration into a pipette tip and by vortexing, the TWA-matrix suspension was centrifuged at 14,000 rpm for 5 min to pellet the remaining solid matrix. The supernatant was carefully removed and diluted with 450 μL of isopropanol. This final ultra-thin layer solution was spread across the sample plate with the side of a pipette and allowed to dry. Typically, 25 μL of ultra-thin layer solution was enough to cover most of a 100-spot Voyager sample plate or a quarter or 384-spot Bruker sample plate. Finally, the dried matrix was gently wiped off the plate by a lint-free wipe, leaving a barely visible matrix layer.

The nanodisc matrix solution was prepared in a manner similar to the ultra-thin layer matrix solution. First, a 2:1:3 *v/v/v* formic acid/water/isopropanol (FWI) solution was prepared and allowed to sit overnight (see [17]). Then, 150 μL of FWI solution was added to 10 mg of solid matrix. This suspension was mixed thoroughly by pipette aspiration and by vortexing. It was then centrifuged at 14,000 rpm for 5 min, and the supernatant was removed. Nanodisc samples were diluted 1:20 in this supernatant FWI-matrix solution, and 0.3 μL were spotted on the ultra-thin layer plate. After 10–15 s, a homogenous polycrystalline layer had formed across the spot, and the remaining solution was removed from the plate by aspiration through a vacuum line attached with a pipette tip. Each spot was washed for several seconds with a 2- μL drop of cold 0.1% TFA in water, which was also removed by aspiration. Both 4HCCA and SA matrices were explored, but SA was used in the majority of studies since it showed superior membrane protein molecular ion peaks.

MALDI-TOF mass spectrometry

MALDI-TOF mass spectrometry was performed on an Applied Biosystems Voyager-DE STR MALDI-TOF mass spectrometer operating at 25 kV accelerating voltage in linear positive ion mode with delayed extraction and a 337 nm nitrogen laser with 3 ns pulses. Laser power was optimized to suit each matrix and varied minimally from

sample to sample. Spectra were averaged over 1,000 shots and were analyzed, smoothed, and baseline-corrected in Voyager Data Explorer 4.0.0.0 software.

Depth-probing MALDI experiments and MALDI mass spectrometry imaging were performed on a Bruker UltrafleXtreme MALDI-TOF/TOF mass spectrometer operating with in linear positive ion mode with pulsed ion extraction. The proprietary Smartbeam™-II laser was capable of varying repetition rates from 1 to 1,000 Hz. Spectra were baseline-corrected, smoothed, and analyzed in FlexAnalysis (Bruker Daltronics). MALDI imaging was performed with the FlexImaging software pack (Bruker Daltronics).

Two different depth probing MALDI experiments were performed. In the first experiment, the laser was rastered across the sample in a regular pattern with 150 μm steps, collecting just one laser shot at each raster point. After rastering across the sample once, the pattern was repeated in the same manner so that the laser fired on the same raster points a second time. The spectra from each pass were summed to give the first shot and second shot spectra. This is referred to as a first-shot MALDI experiment.

Since the first-shot MALDI experiment using MALDI imaging was relatively time-intensive, a second technique was used to access deeper depths. In this second experiment, the laser was rastered over the sample in a random walk pattern. The number of shots taken at each specific raster point was changed to include 1, 3, 5, and 10. Spectra were then summed and compared based on how many shots the laser had taken at each raster point. The resultant spectra indicated the average composition of the first shot, shots 1 through 3, shots 1 through 5, and shots 1 through 10.

Coffee ring spots were formed in the same way the optimized ultra-thin layer spots were formed except that the

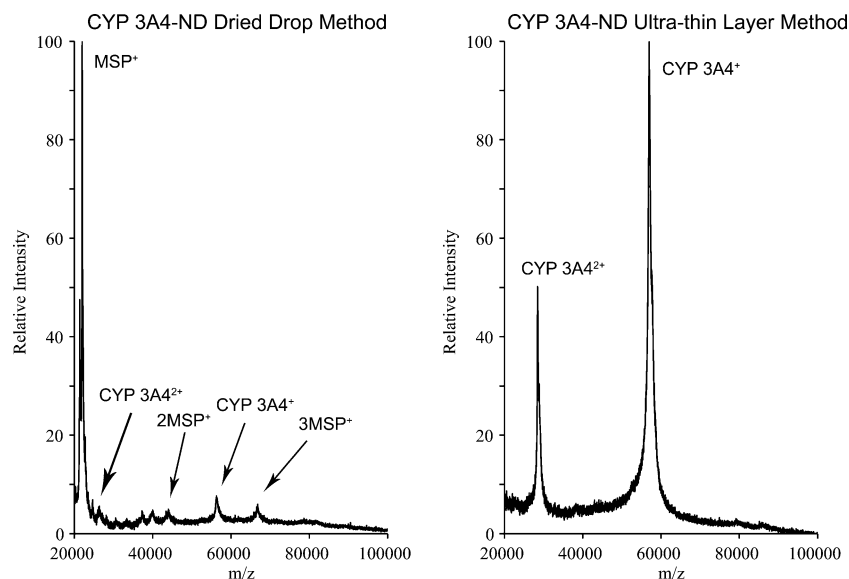
solution was aspirated off the sample plate immediately after it had been spotted. To image the coffee ring spots, the laser was rastered across the image in 75 μm steps collecting 200 shots at each raster point at a 1 kHz laser firing rate. Spectra were baseline-corrected and smoothed in FlexAnalysis and imaged in FlexImaging.

Results and discussion

The most common method of sample preparation for proteins and peptides is the dried-drop method. The dried-drop method involves simply mixing the analyte with matrix and spotting the mixture on the sample plate to dry. To test the applicability of the dried-drop method to membrane proteins in nanodiscs, we began by examining cytochrome P450 3A4 in nanodiscs. Cytochrome P450 3A4 is the most abundant human P450 and is involved in xenobiotic metabolism. This protein is monotonically embedded in the lipid bilayer and has been extensively studied in nanodiscs (see [32] and references therein). With the dried-drop method, strong membrane scaffold protein (MSP) signal was seen at 22.0 kDa with MSP dimers and trimers appearing at 44.0 and 66.0 kDa, respectively (see Fig. 2). Comparatively small CYP 3A4 peaks were observed at 57.0 and 28.5 kDa corresponding to the mono- and di-cation species. The strong signal from MSP and weak signal from CYP 3A4 is problematic for analysis of the membrane protein inside the nanodiscs.

To improve the membrane protein signal, we optimized the ultra-thin layer sample preparation technique, originally developed for membrane proteins in detergent [24], for membrane proteins in nanodiscs. The MALDI-TOF spectra of CYP 3A4 in nanodiscs using dried-drop method and

Fig. 2 Comparison of the MALDI-TOF spectra with sinapinic acid matrix for CYP 3A4 in nanodiscs using the dried-drop method (*left*) and the ultra-thin layer method (*right*). With the dried-drop method, strong MSP peaks are observed with monomers, dimers, and trimers at 22.0, 44.0, and 66.0 kDa, respectively. CYP 3A4 peaks are relatively weak with peaks at 57.0 and 28.5 kDa. With the ultra-thin layer method, MSP is absent, and the mono- and di-cation CYP 3A4 peaks are enhanced



ultra-thin layer method are compared in Fig. 2. Applied to CYP 3A4 in nanodiscs, the ultra-thin layer method showed almost no detectable MSP signal and a roughly two- to fourfold signal enhancement of the CYP 3A4 peaks.

We used two different matrices in conjunction with the ultra-thin layer method to determine their utility with nanodisc samples. Each matrix showed MSP elimination and enhanced CYP 3A4 peaks. Similar to previous studies [24], α -cyano-4-hydroxycinnamic acid (4HCCA) showed more polycationic species than sinapinic acid (SA). This is beneficial in studies where high mass accuracy is needed, and multiple ion peaks can be averaged to get more precise mass values. On the other hand, SA showed much better signal from the singly and doubly charged CYP 3A4 but fewer species of higher charge. This matrix will be useful for mixtures or other applications where simpler spectra or singly charged species are desirable. Since it showed better singly charged protein peaks, sinapinic acid was used in the remaining studies.

While it was not surprising that the ultra-thin layer method improved the membrane protein signal, given its success in detergent-based systems, the discovery that the ultra-thin layer method nearly completely eliminated the MSP signal was serendipitous and led us to wonder about the ultimate fate of the MSP. Several experiments were performed to characterize the spots formed by the ultra-thin layer method. In the first simple experiment, we manually moved the laser around the spot to probe various regions of the spot. It was observed that the CYP 3A4 signal had a fairly homogeneous lateral distribution with few “hot spots.” Moreover, MSP was not localized to any region of the spot.

To investigate the depth profile of the sample spots, MALDI experiments, including first-shot MALDI, were performed utilizing the laser as a depth probe. Spectra were sorted based on the number of times a laser had fired on a specific location [33]. By examining how the mass spectra change as the laser probed different depths, these experiments showed the vertical distribution of protein within the polycrystalline matrix spot. In both experiments, there were no significant changes in spectra as the laser probed deeper into the spot. The distribution of CYP 3A4 was vertically homogenous throughout the spot, and no MSP was observed throughout the sample. Thus, the enhanced membrane protein signal is not due to an upconcentration of membrane protein at the surface or in any other region of the spot, and the lack of MSP signal is not likely due to a sequestration within the matrix crystal.

One of the important steps in ultra-thin layer sample preparation is the removal of excess solution following the co-crystallization of matrix and protein on the ultra-thin layer. The solution is removed by vacuum aspiration before the sample dries completely. This step and the subsequent

rinse with 0.1% TFA in cold water serve to remove salts, lipids, and other contaminants [24] and greatly improves the quality of the spectra. In the process of optimizing the method, it was observed that the amount of time given for the spot to crystallize before aspiration of the solution had significant effects on the MSP signal. When the spots were allowed to dry for 10 s or more before aspiration of the remaining solution, the crystal layer looked very homogeneous and showed almost no MSP signal across the entire spot.

However, if the solution was immediately aspirated off the surface, a coffee ring drying pattern was observed with a thicker layer of matrix around the edges and a thin matrix deposit in the middle. MALDI mass spectrometry imaging of these rapidly dried spots showed almost no MSP present in the thick layer around the ring (similar to the optimized ultra-thin layer spectra), strong MSP peaks in the thin deposits in the middle (similar to the dried-drop spectra), and a mixed spectra showing moderate MSP and protein peaks in the region of the edge directly adjacent to where the vacuum aspirator removed the solution. The MALDI image of a coffee ring spot is shown in Fig. 3 with the integrated intensity of the 57.0 kDa CYP 3A4 peak in blue and the integrated intensity of the 22.0 kDa MSP peak in red. The region of purple near the top indicates the mixed spectra formed near the aspirator.

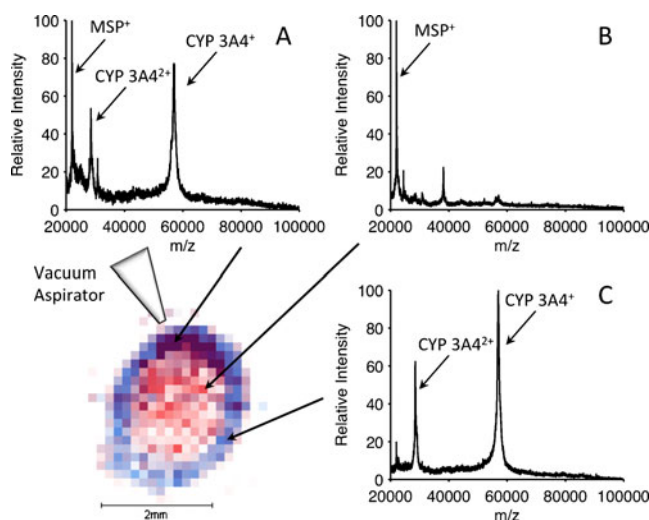


Fig. 3 Mass spectra and MALDI mass spectrometry image of a rapidly dried coffee ring spot of CYP 3A4 in nanodiscs using the ultra-thin layer method and sinapinic acid. In the region adjacent where the vacuum aspirator had suctioned the drop off the chip, a mixed spectrum is observed showing both MSP and CYP 3A4 (A). In the middle, the MSP peak is strong, and CYP 3A4 peaks are weak (B). Around the edge, CYP 3A4 peaks are prevalent, and MSP is suppressed (C). The MALDI-MS image shows the integrated intensity of the CYP 3A4 peak at 57.0 kDa in blue and the integrated intensity of the MSP peak at 22.0 kDa in red. The image mirrors spectra C, B, and A with a blue CYP 3A4 ring around the edge of the spot, red in the middle of the spot, and mixed purple adjacent to the aspirator

The heterogeneous spots observed with fast-drying by aspiration provide a model for understanding the MSP elimination that is similar to previous models of differential protein detection [34]. The 2:1:3 formic acid/water/isopropanol solvent breaks up the nanodisc and solvates the protein, lipids, and MSP. The ultra-thin layer on the surface of the sample plate forms a layer of seed crystals, which nucleates crystal formation on the surface. Since sinapinic acid crystals have hydrophobic faces [35], a partitioning occurs between the hydrophobic matrix and the solvent. With longer drying times, MSP partitions into the solvent while the membrane proteins partition onto the growing polycrystalline film and are co-incorporated homogeneously as the solvent evaporates and the crystal layer grows. MSP remains in solution and is removed with the solution by aspiration. Any residual MSP is washed away with the TFA rinse.

Under conditions of fast drying, there is a brief period of time before aspiration begins where the partitioning observed with normal drying conditions occurs. In this short period of time, the membrane protein partitions into the coffee ring layer forming at the exterior of the drop. MSP remains partitioned in solution. Vacuum aspiration, however, causes much faster evaporation of the drop. Thus, MSP does not have time to partition freely into solution and becomes trapped in precipitating matrix crystals. Kinetic limitations cause the formation of crystals that resemble the dried-drop method more closely than the ultra-thin layer method. MSP deposits in this way in the center of the spot and over top of the existing coffee ring directly adjacent to the site of aspiration as the drop is pulled from the center, over the coffee ring, and into the aspirator.

To demonstrate that the dramatic elimination of MSP and enhancement of membrane protein peaks is general for multiple types of membrane proteins, we applied this method to nanodiscs containing membrane proteins of different sizes and membrane topologies. Cytochrome P450 reductase (CPR) is a membrane-associated redox partner with many P450 enzymes. Structurally, CPR is bound to the membrane by a single transmembrane helix. Thus, it is primarily a soluble protein with a small transmembrane anchor (Fig. 1). Figure 4 shows the MALDI-TOF spectrum of CPR in nanodiscs with the ultra-thin layer method. There are peaks at 77.7, 39.0, and 25.9 kDa corresponding to the mono-, di-, and tri-cation of the full-length protein. A triply charged dimer species appears at 51.4 kDa. Dimers also appear weakly in spectra from rhodopsin and CYP 3A4.

The method was further tested on rhodopsin, a member of the G protein-coupled receptor family of integral membrane proteins. Rhodopsin is a polytopic transmembrane protein and is more completely embedded in the membrane than either CYP 3A4 or CPR (Fig. 1). Previously, strong MSP peaks and weak rhodopsin peaks were observed in SAMDI spectra of rhodopsin in nanodiscs [14]. MALDI-TOF mass spectra of rhodopsin in nanodiscs using the ultra-thin layer method showed rhodopsin peaks at 41.7 and 20.9 kDa for the mono- and di-cation species with almost no MSP present (see Fig. 4). Dimeric rhodopsin is also observed around 83 kDa. The successful application of the ultra-thin layer method to structurally diverse proteins ranging from integral transmembrane to monotonically embedded to membrane anchored proteins demonstrates the general applicability of this method for MALDI of membrane proteins in nanodiscs.

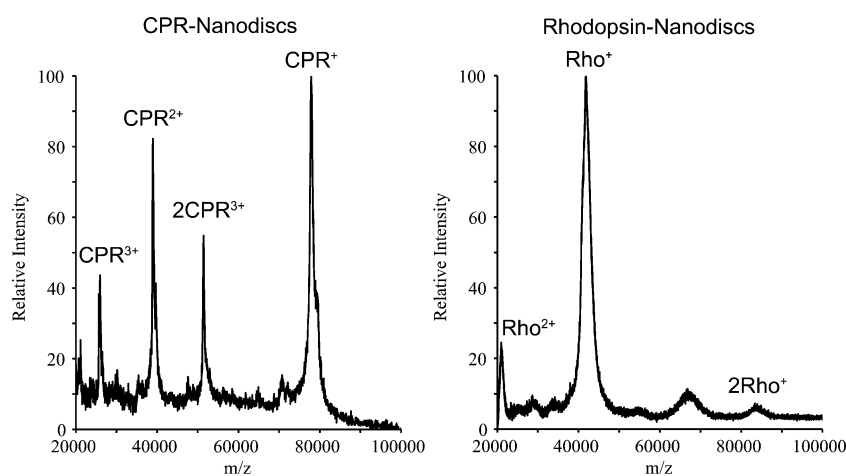


Fig. 4 The MALDI-TOF mass spectrum of cytochrome P450 reductase in nanodiscs using the ultra-thin layer method is shown on the *left*. The mono-, di-, and tri-cation peaks are observed at 77.7, 39.0, and 25.9 kDa, respectively. A dimer with three charges appears at 51.4 kDa. The MALDI-TOF mass spectrum of rhodopsin in

nanodiscs using the ultra-thin layer method is shown on the *right*. Mono- and di-cation peaks appear at 41.7 and 20.9 kDa. Dimers appear around 83 kDa. An unidentifiable peak around 66 kDa may be MSP trimers or some other aggregate species

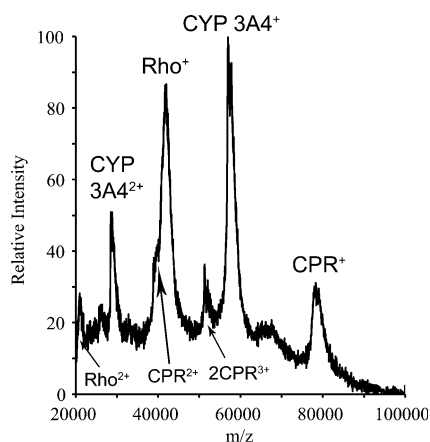


Fig. 5 Mass spectrum of a mixture of CYP 3A4 nanodiscs, CPR nanodiscs, and rhodopsin nanodiscs. Peaks appearing in Figs. 2 and 4 are all present, and MSP is still nearly absent despite representing a larger fraction of total protein

One of the potential applications of MALDI mass spectrometry of nanodiscs is in the identification of specific membrane proteins binding in a mixture or complex. Mass spectrometry has previously been used to identify binding partners from surface plasmon resonance assays [36], immunoprecipitation assays [13], and the SAMDI assay described above [14]. While mass spectrometry of full-length proteins will not likely yield the specificity required to identify unknown proteins, it allows fast identification of known proteins from a heterogeneous mixture in experiments where multiple proteins are interacting. For heterogeneous populations of membrane proteins in nanodiscs, MALDI with the dried-drop method will be severely hampered by strong MSP signal as the ratio of each protein to MSP will be smaller in mixtures. To examine the efficacy of the ultra-thin layer method on heterogeneous nanodisc samples, mixtures were prepared by combining the CYP 3A4, CPR, and rhodopsin nanodisc samples described above. MALDI mass spectrometry using the ultra-thin layer method demonstrated that MSP peaks were suppressed in the mixture, and CYP 3A4, CPR, and rhodopsin could all be seen in the spectrum (see Fig. 5). These results demonstrate the ability to identify multiple and diverse membrane proteins in nanodiscs and open the door for future applications involving larger protein complexes.

Conclusion

We have detailed a method for studying membrane proteins in nanodiscs using MALDI-TOF mass spectrometry. The dried-drop method of sample preparation showed strong signal from the membrane scaffold protein belt and weak signal from membrane proteins inside nanodiscs. Using the

ultra-thin layer method [24], we observed enhanced signal from membrane proteins in nanodiscs and a nearly complete elimination of MSP signal. These results have been demonstrated for a variety of membrane proteins in nanodiscs, including mixtures of proteins. The ability to efficiently detect membrane proteins in nanodiscs with MALDI mass spectrometry enables the development of future mass spectrometry experiments for functional and structural studies.

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